IOSR Journal Of Pharmacy www.iosrphr.org
(e)-ISSN: 2250-3013, (p)-ISSN: 2319-4219
Volume 9, Issue 1 Version. I (January 2019), PP. 50-61

Simultaneous spectrophotometric determination of Levofloxacin and Azithromycin using π-acceptors as analytical reagents

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Abstract: Two sensitive and precise spectrophotometric methods have been developed for the simultaneous determination of levofloxacin and azithromycin in pure mixture and in pharmaceutical binary dosage forms. A new concept of area under curve (AUC) is proposed for simultaneous estimation of two drugs by these methods. Method A involves the use of DDQ (2,3-Dichloro-5,6-dicyano-1,4-benzoquinone) as analytical reagent and the AUC between 390nm and 690nm for DDQ was used for determination. Method B involves the use of *p*-CA (*p*-Chloranilic acid: 2,5-Dichloro-3,6-dihydroxy-1,4-benzoquinone) as an analytical reagent and the AUC between 400nm and 700nm for *p*-CA was used for determination. The methods developed and construction of calibration curves using two analytical reagents viz., DDQ and *p*-CA are described. Optical and analytical parameters for the individual and simultaneous determination of levofloxacin and azithromycin using AUC are tabulated. The methods have been validated and compared with HPLC methods in terms of standard deviation, t-test and F-test.

Keywords - Spectrophotometry, Simultaneous estimation, AUC, Levofloxacin, Azithrmycin, Azitech-Le tablet, DDQ, *p*-CA, CT Complex, Validation

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Date of Submission: 22-01-2019 Date of acceptance: 05-02-2019

I. INTRODUCTION

The present study is aimed at the development of two sensitive and simple spectrophotometric methods for the simultaneous determination of Levofloxacin and Azithromycin in pure mixture and pharmaceutical binary dosage forms using π-Acceptors as analytical reagents.

1.1 Levofloxacin

Levofloxacin (Fig 1), a newer member of quinolones, is used in the treatment of Multidrug Resistant (MD) tuberculosis [1]. It is the L- isomer of ofloxacin existing commercially as the hemihydrate. Chemically, it is (-)-(S)-9-fluoro- 2,3-dihydro-3-methyl - 10 - (4-methyl-1-piperazinyl) -7- oxo - 7H – Pyrido [1,2,3-de] - 1,4-benzoxazine - 6-carboxylic acid, hemihydrate. It is greatly effective against both Gram-negative and Gram-positive bacteria that are resistant to other antibacterials[2-4].

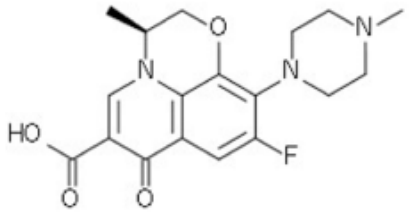

Fig 1: Levofloxacin

Levofloxacin (LEV) is the quinolone of choice for airway infections, being active against several types of pathogens[5-8]. Various analytical methods have been reported in scientific literature for the analysis of Levofloxacin in pharmaceutical formulation and/or biological fluids including HPLC with UV detection[9], Vibrational Spectroscopy[10], Spectrofluorimetry[11], Colorimetric Spectrophotometry[12], Spectro photometry by ion-pair complex[13, 14], UV Spectrophotometry[15] and Capillary Electrophoresis[16].

1.2 Azithromycin

Azithromycin (Fig 2) (AZI) is a semi synthetic macrolide antibiotic with a 15-membered azalactone ring. It is derived from erythromycin. however, it differs chemically from erythromycin in that a methyl-substituted nitrogen atom is incorporated into the lactone ring..

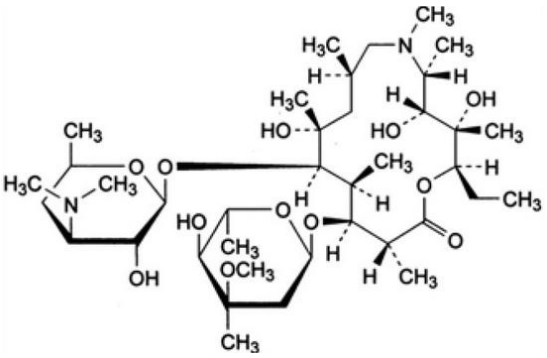

Fig 2: Azithromycin

Like erythromycin, it appears to bind to the same receptor, 50 S ribosomal subunits of susceptible bacteria and suppresses protein synthesis. It is used primarily to treat various bacterial infections, such as aerobic gram-positive microorganisms and aerobic gram-negative microorganisms. The incorporation of the nitrogen into the ring significantly alters the chemical, microbiological and pharmacokinetic properties of Azithromycin. It exhibits a more extensive spectrum of activity, greater acid stability and more favorable pharmacokinetic parameters than erythromycin[17].

Several methods have been developed for the determination of Azithromycin in pharmaceutical dosage forms. These methods include HPLC and microbiological methods. Chromatographic separation is one of the essential and powerful components of the most quantitative analyses and HPLC is currently the most versatile tool which satisfies the needs for an optimum separation[18]. Azithromycin has been analyzed by HPLC using fluorescence[19-21], electrochemical (using amperometric and coulometric detectors)[22, 23] and mass spectrometry detector[24-27] for quantification in bulk material and pharmaceutical dosage forms. Fluorescence detection requires complicated sample pretreatment involving pre-column derivatization of the analyte. Assay procedures making use of electrochemical detection is often very time consuming, both in the sample preparation steps and the chromatography. The United States Pharmacopeia (USP) method[28] describes a high *p*H mobile phase (*p*H 11) as well as a specific column (Gamma alumina) which is quite expensive and difficult to obtain commercially as many of the column manufacturers do not supply this column. Also, the USP method employs amperometric electrochemical detection, which is not available in many laboratories. Mass spectrometry methods may have the highest sensitivity, but the determination process is complex[29]. Development of a Simple RP-HPLC-UV method for determination of Azithromycin in bulk and pharmaceutical dosage forms as an alternative to the USP method has been reported[30], RP-HPLC method was developed and validated for simultaneous determination of Azithromycin and Levofloxacin in tablet dosage form[31, 32] and in their pure form[33]. Simultaneous determination of azithromycin and levofloxacin in pharmaceuticals by charge transfer complexation with alizarin red S using an absorption-factor method has been recently reported[34]. The literature survey revealed that methods of analysis of binary mixtures use direct UV spectra of the drugs and no foreign analytical reagent is used. For example, DDQ (2,3-Dichloro-5,6-dicyano-1,4-benzoquinone), I_2, *p*-CA (2,5-Dichloro-3,6-dihydroxy-1,4-benzoquinone) and TCNE (Tetracyano ethylene) are used as analytical reagents for mono dosage forms, but are not used for the analysis of binary mixtures.

II. MATERIALS AND METHODS

2.1 Instruments

The UV-Vis spectra of the study have been recorded on SHIMADZU 140 double beam spectrophotometer and also on ELICO SL 210 UV-Visible double beam spectrophotometer using quartz cells of 10 mm path length. An Elico model Li-120 *p*H meter was used for *p*H measurement.

2.2 Materials

DDQ (2,3-Dichloro-5,6-dicyano-p-benzoquinone) was obtained from SD Fine Chemicals. It was recrystallized twice from 3:1 mixture of chloroform and benzene. p-CA (P-Chloranilic acid) supplied by Rolex, Mumbai was used without further purification. HPLC grade acetonitrile was used throughout the work. The drug mixture analysed was procured from Dr. Reddy's laboratories, Hetero Drugs Private Ltd, Kekule Pharma

Limited, Srini Pharmaceuticals Ltd. and Symed Laboratories Ltd. as gift samples. All these firms are located in and around Hydeabad, Telangana.

2.3 Methods and Calibration

2.3.1 Method A

This method is developed for the simultaneous estimation of drugs in a binary mixture using DDQ (2,3-Dichloro-5,6-dicyano-1,4-benzoquinone) as an analytical reagent. Into a series of 10ml of flasks, different aliquots (1-9ml) of levofloxacin were taken and 1ml of DDQ was added, remaining volume was made up with solvent (Acetonitrile). The contents were shaken well and UV–Vis spectra were recorded. The OD at 480, 540 and 580nm for DDQ anion were noted. The areas under the curve (AUC) between 390nm and 690nm for DDQ were determined from the spectra. AUC_x was plotted against concentration of levofloxacin. From the slope of the plot K_x was determined. Similarly, analogous experiments were repeated for determination of K_y for azithromycin.

Stock solution of mixture of levofloxacin and azithromycin was prepared with same ratio as in tablet formulations. Form the stock, 1-9ml of mixture of drugs were taken into series of standard flasks and 1ml of reagent DDQ was added. Remaining volume was made up with solvent (Acetonitrile). The contents were shaken well. UV-Vis spectra were recorded. The OD at 480,540 & 580 for DDQ anion were noted. AUC_{mix} was plotted against either Cx or Cy for calibration.

2.3.2 Method B

This method is developed for the simultaneous estimation of drugs in a binary mixture using *p*-CA (*p*-Chloranilic acid: 2,5-Dichloro-3,6-dihydroxy-1,4-benzoquinone) as an analytical reagent. Into a series of 10ml of flasks, different aliquots (1-9ml) of one drug were taken and 1ml of *p*-CA was added, remaining volume was made up with solvent (Acetonitrile). The contents were shaken well and UV–Vis spectra were recorded. The OD at 540nm for *p*-CA anion were noted. The areas under the curve (AUC) between 400nm and 700nm for *p*-CA were determined from the spectra. AUC_x is plotted against the concentration of drug. From the slope of the plot K_x was determined. Similarly, analogous experiments were repeated for determination of K_y for another drug.

Stock solution of mixture of drugs was prepared with same ratio as in tablet formulations. Form the stock, 1-9ml of mixture of drugs were taken into series of standard flasks and 1ml of reagent, *p*-CA was added. Remaining volume was made up with solvent (Acetonitrile). The contents were shaken well. UV-Visible spectra were recorded. The OD at 540nm for *p*-CA anion was noted. AUC_{mix} was plotted against either Cx or Cy for calibration.

III. RESULTS AND DISCUSSION

p-CA for example, is an analytical reagent and produces a band at 540nm for *p*-CA anion and is independent of the drug. It is also expected to interact with both the drugs in mixture and exhibits band at 540 nm. As the extent of interaction is different in mixture, it is possible to analyze the concentration of each although the analytical wavelength is same. This prompted the author to give a thought in these lines. For the quantification, generally optical density at λ_{max} is measured against concentration of drug for calibration purpose. The authors area under curve (AUC) is more appropriate than the optical density. The author proposes to measure the area under the curve for individual drugs as well as the mixture in a constant ratio of concentration as in the formulations.

AUC (Area under curve in mixture) = $AUC_X + AUC_Y$

where X and Y are two drugs in the binary mixture

but AUC of X α C_X

and AUC of Y α C_Y

$$AUC_X = K_X C_X$$

$$AUC_Y = K_Y C_Y$$

$$AUC_{mix} = K_X C_X + K_Y C_Y \quad \ldots\ldots (1)$$

Dividing both sides of equation by $K_X C_X$

$$\frac{AUC_{mix}}{K_x C_x} = 1 + \frac{K_Y C_Y}{K_x C_x}$$

But $\frac{K_Y C_Y}{K_x C_x} = K$ (Constant)

$$\frac{AUC_{mix}}{K_x C_x} = 1 + K$$

$AUC_{mix} = (1 + K)K_XC_X$

$AUC_{mix} = (K_X + K, K_X)C_X$ ……(2)

Similarly

$AUC_{mix} = K_XC_X + K_YC_Y$

Dividing both sides with K_YC_Y

$$\frac{AUC_{mix}}{K_YC_Y} = 1 + \frac{K_xC_x}{K_YC_Y}$$

$$\frac{K_xC_x}{K_YC_Y} = K \text{ (Constant)}$$

$AUC_{mix} = (1 + K)K_YC_Y$ ……(3)

$AUC_{mix} = (K_Y + K. K_Y)C_Y$ ……(4)

The equations 2 and 4 imply that AUC_{mix} is either proportional to C_x or C_Y

By determining the AUC_{mix} for a mixture of drugs having constant ratio it is possible to construct the calibrations to find the individual concentrations of drugs in a binary mixture.

Into a series of 10ml of flasks, different aliquots (1-9ml) of drug Levofloxacin were taken and 1ml of DDQ or p-CA was added, remaining volume was made up with solvent acetonitrile. The contents were shaken well and UV–Vis spectra were recorded. The OD at 540nm for *p*-CA anion and 480, 540 and 580nm for DDQ anion were noted. The area under the curve (AUC) between 390nm and 650nm for DDQ and between 400nm and 700nm for *p*-CA were determined from the spectra (Fig. 3 and Fig. 4). The plots of AUC_x vs concentration of Levofloxacin with DDQ and *p*-CA are shown in Fig. 5 and Fig. 6. From the slope of the plots K_x was determined. In the same way, analogous experiments were repeated for determination of K_y for Azithromycin (Fig. 7, Fig. 8, Fig. 9 and Fig. 10).

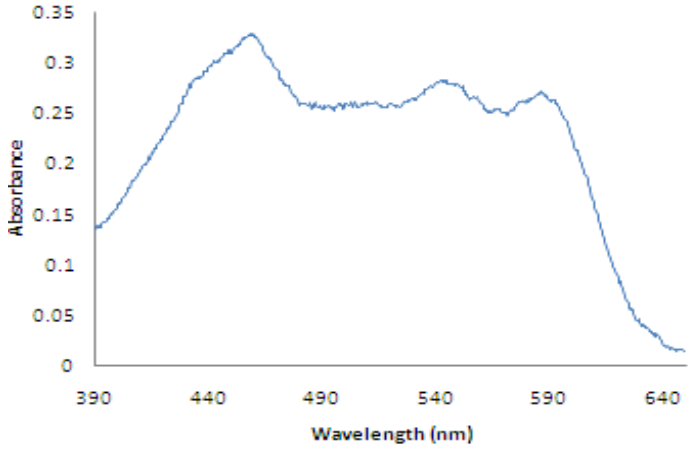

Fig 3: Charge transfer spectrum of Levofloxacin with DDQ

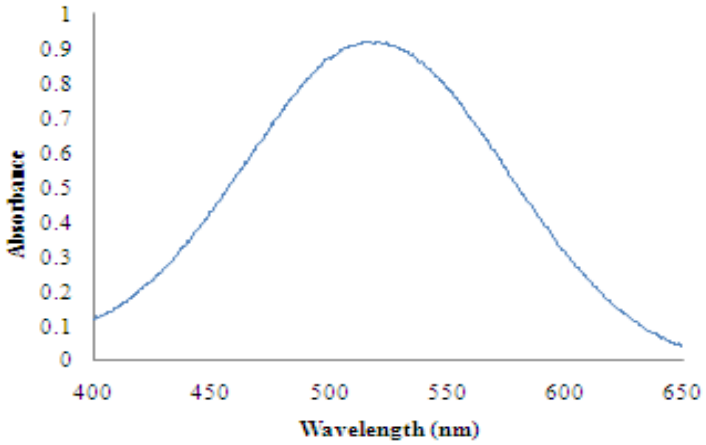

Fig 4: Charge transfer spectrum of Levofloxacin with p-CA

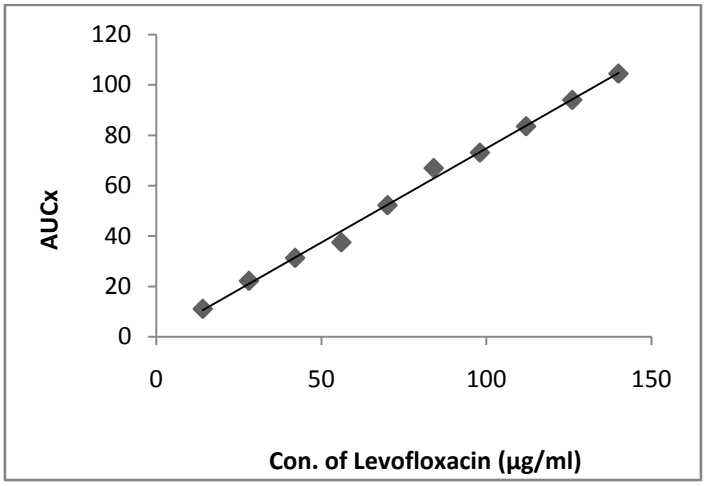

Fig 5: Plot of AUC vs Con. Levofloxacin -DDQ

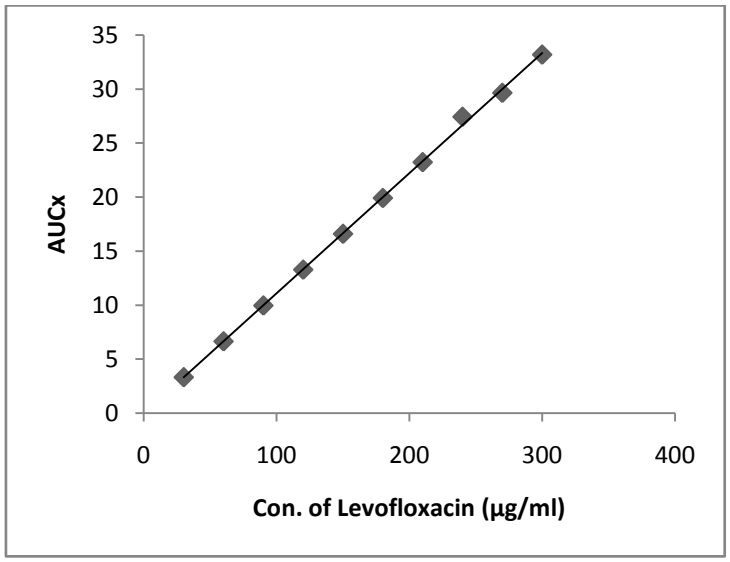

Fig 6: Plot of AUC vs Con. of Levofloxacin -p-CA

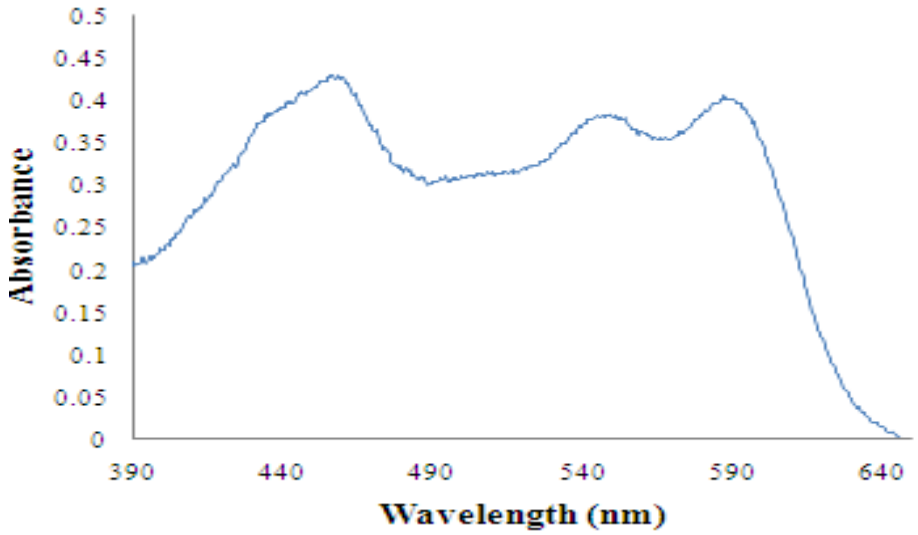

Fig 7: Charge transfer spectrum of Azithromycin with DDQ

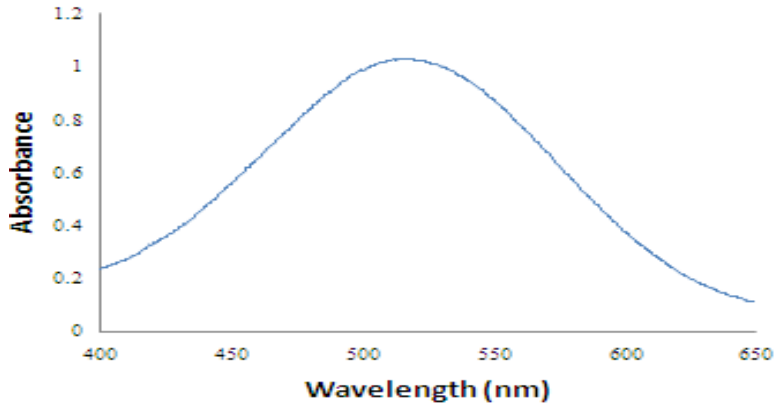

Fig 8: Charge transfer spectrum of Azithromycin with p-CA

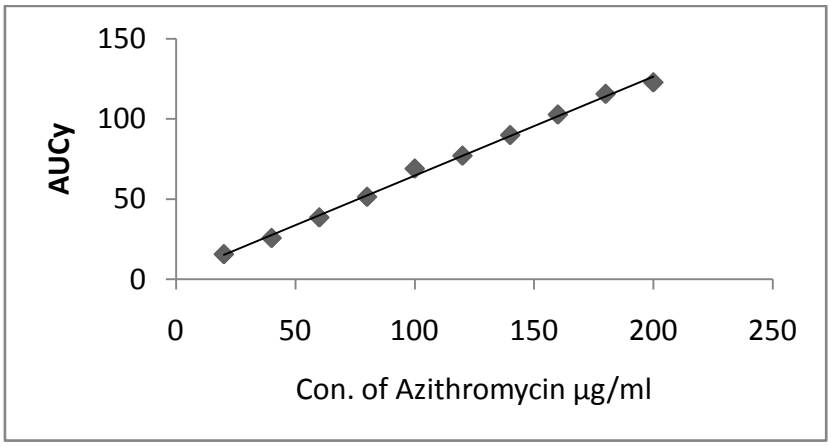

Fig 9: Plot of AUC vs Con. of Azithromycin-DDQ

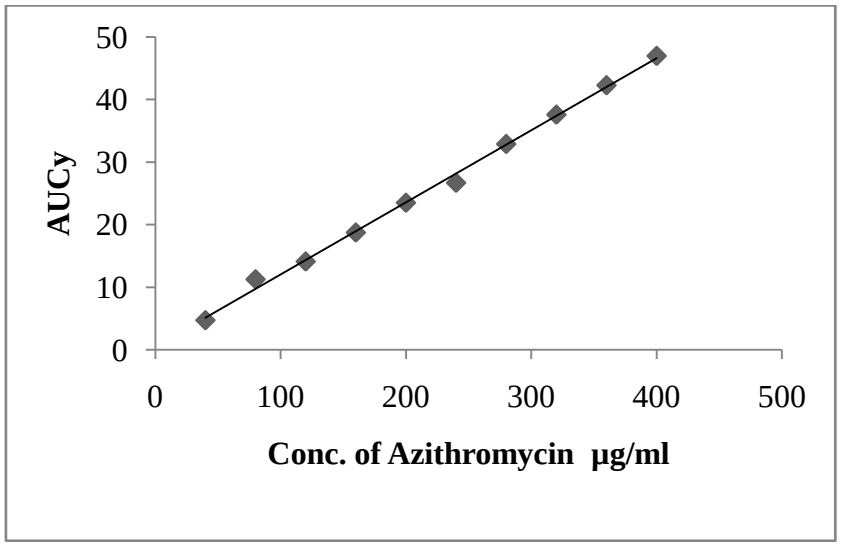

Fig 10: Plot of AUC vs Con. of Azithromycin-p-CA

Stock solution of mixture of drugs was prepared with same ratio as in tablet formulations. From the stock 1-9ml of mixture of drugs were taken into series of standard flasks and 1ml of reagent DDQ or *p*-CA was added. Remaining volume was made up with solvent (Acetonitrile). The contents were shaken well. UV-Visible spectra were recorded (Fig. 11 and Fig. 12). The OD at 540nm for *p*-CA anion and 480, 540 & 580 for DDQ anion were noted. AUC_{mix} was plotted either Cx or Cy (Fig. 13 and Fig. 14).

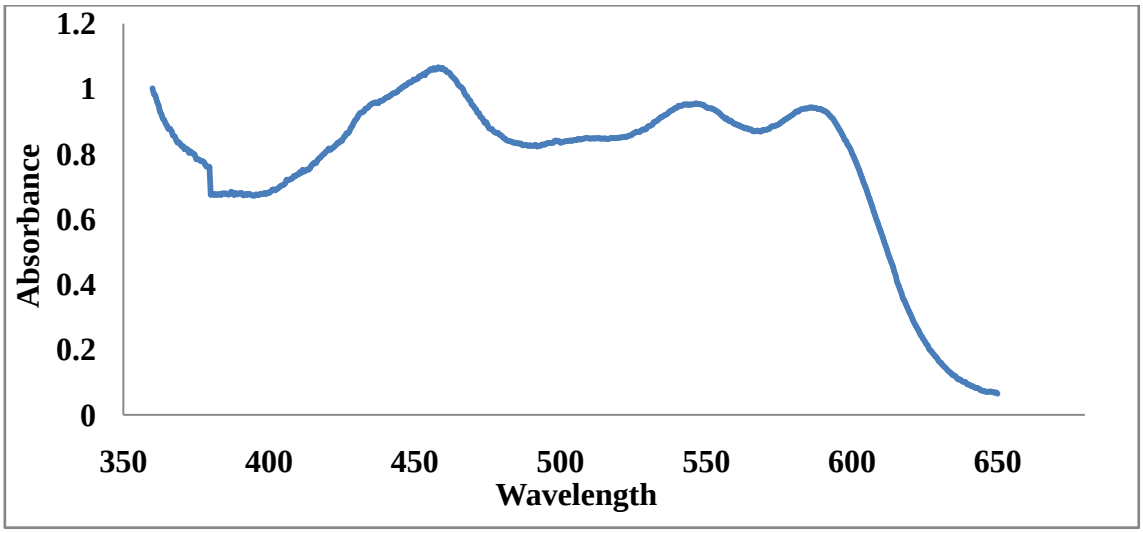

Fig 11: Charge transfer spectrum of LEV+AZI with DDQ

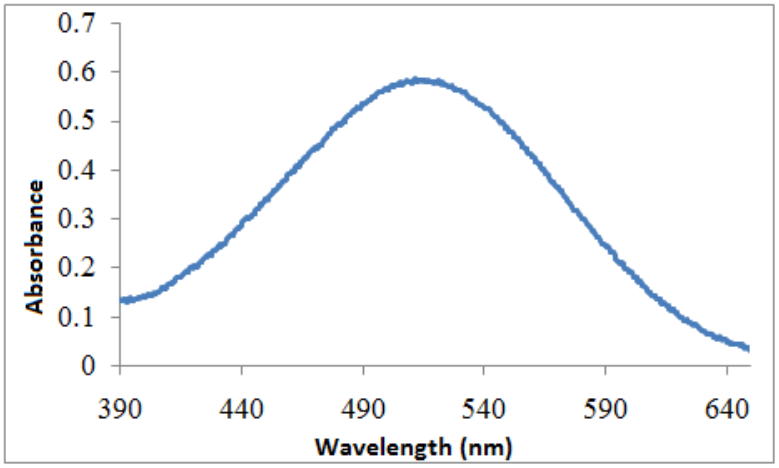

Fig 12: Charge transfer spectrum of LEV+AZI with p-CA

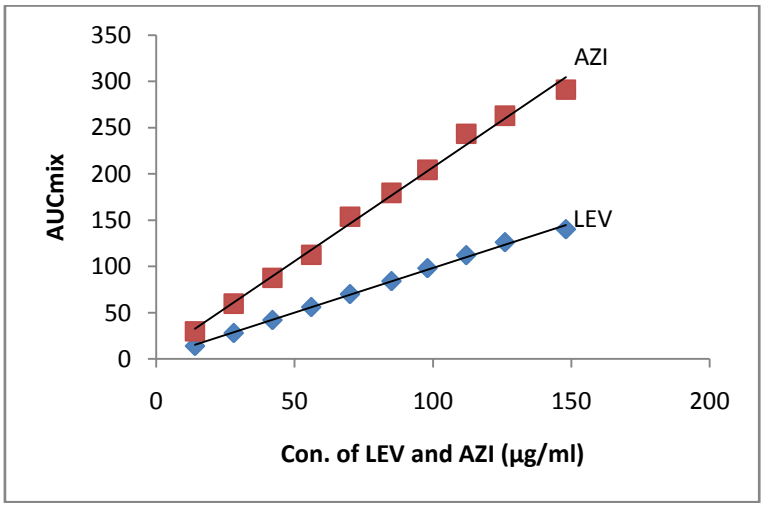

Fig 13: Plot of AUCmix vs Con. of LEV & AZI-DDQ in pure form

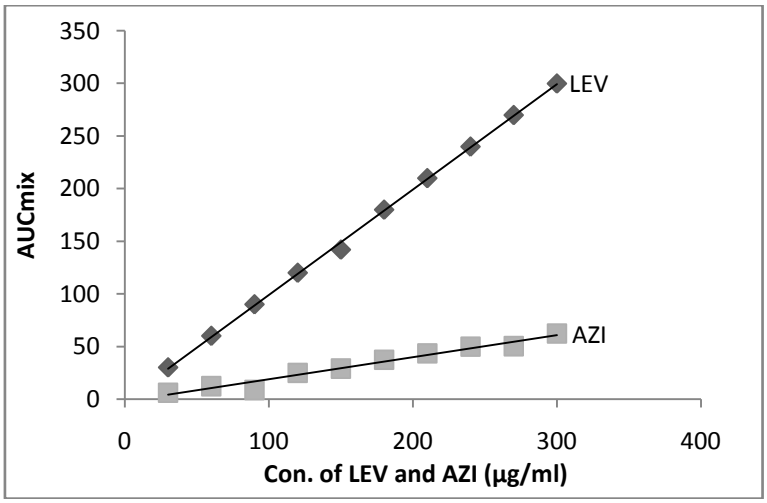

Fig 14: Plot of AUCmix vs Con. of LEV & AZI--p-CA in pure form

The optical characteristics and statistical data for the regression equation of the proposed method for the determination of individual drugs (Levofloxacin and Azithromycin) are presented in Table 1 and in synthetic mixture in the ratio of 1:1 of drugs as in tablets using area under curve (AUC) are presented in Table 2.

Table 1: Optical and analytical parameters for the individual determination of Levofloxacin and Azithromycin using Area Under Curve

Parameters	**DDQ**		***p*-CA**	
λ Lower and λ Higher for AUC	390nm – 650nm		400nm – 700nm	
Range of concentrations of drugs (µgmL^{-1})	**Levofloxacin**	**Azithromycin**	**Levofloxacin**	**Azithromycin**
	10-200	12-225	30-300	40-500
Slope	2.009	0.797	0.129	0.798
Intercept	-0.946	0.406	-0.302	0.112
Correlation coefficient	0.998	0.999	0.998	0.999
Residual intercept	0.6087	0.2898	0.1172	0.967
LOD	1	1.4	3	5.0
LOQ	3.3	4.62	9.9	16.5

Table 2: Optical and analytical parameters for the simultaneous determination of Levofloxacin and Azithromycin in synthetic mixture in the ratio of 1:1 of drugs as in tablet using Area Under Curve

Parameters	**DDQ**		***p*-CA**	
λ Lower and λ Higher for AUC	390nm – 650nm		400nm – 700nm	
Range of concentrations of drugs (µgmL^{-1})	**Levofloxacin**	**Azithromycin**	**Levofloxacin**	**Azithromycin**
	14-140	14-140	30-300	30-300
Slope	1.000	2.104	1.000	0.199
Intercept	0.133	0.474	-0.382	0.413
Correlation coefficient	0.999	0.997	0.999	0.99
Residual intercept	0.2278	0.5778	0.9090	0.1809
LOD	1.4	1.4	3.0	3.0
LOQ	4.62	4.62	9.9	9.9

Five different solutions of pure drug mixture in the range of calibration curve were selected and the recovery experiments were performed. The recoveries and their relative standard deviations are tabulated in Table 3.

Similarly, different solutions of Azitech-Le tablets (1:1) in the range of calibration curve were chosen and the assay was estimated using the calibration curve (Fig. 15 and 16). The results of the recovery experiments are tabulated in Table 4.

Table 3: Application of proposed methods for the simultaneous determination of Levofloxacin and Azithromycin in the mixture in the ratio of 1:1 of drugs in pure form using Area Under Curve

Taken (μg ml^{-1})				Found (μg ml^{-1})				Recovery (%)			
Levofloxacin		Azithromycin		Levofloxacin		Azithromycin		Levofloxacin		Azithromycin	
DDQ	*p*-CA	DDQ	*p*-CA	DDQ	*p*-CA	DDQ	*p*-CA	DDQ	*p*-CA	DDQ	*p*-CA
14	30	14	30	13.98	30.53	14.16	29.42	99.85	101.76	101.14	98.06
28	60	28	60	28.32	59.68	28.24	60.23	101.14	99.46	100.85	100.38
42	90	42	90	41.88	89.45	41.54	90.88	99.71	99.38	98.90	100.97
56	120	56	120	55.84	121.85	55.65	121.00	99.71	101.54	99.37	100.83
70	150	70	150	71.85	150.64	71.12	151.3	102.64	100.42	101.60	100.86
84	180	84	180	84.23	181.62	84.56	180.54	100.27	100.90	100.66	100.30

SD Proposed method				SD Reference method			
Levofloxacin		Azithromycin		Levofloxacin		Azithromycin	
DDQ	*p*-CA	DDQ	*p*-CA	DDQ	*p*-CA	DDQ	*p*-CA
1.1572	1.0135	1.0551	1.0992	1.0872	1.2212	1.3253	1.1313

t-Test				F-test			
Levofloxacin		Azithromycin		Levofloxacin		Azithromycin	
DDQ	*p*-CA	DDQ	*p*-CA	DDQ	*p*-CA	DDQ	*p*-CA
0.0960	0.2922	0.3576	0.0447	0.8827	1.4519	1.5778	1.0593

Table 4: Application of proposed methods for the simultaneous determination of Levofloxacin and Azithromycin in the mixture in the ratio of 1:1 of drugs in pharmacutical form (Azitech-Le tablets) using Area Under Curve

Taken (μg ml^{-1})				Found (μg ml^{-1})				Recovery (%)			
Levofloxacin		Azithromycin		Levofloxacin		Azithromycin		Levofloxacin		Azithromycin	
DDQ	*p*-CA	DDQ	*p*-CA	DDQ	*p*-CA	DDQ	*p*-CA	DDQ	*p*-CA	DDQ	*p*-CA
14	30	14	30	14.16	29.84	14.23	30.02	101.14	99.46	101.64	100.06
28	60	28	60	27.96	60.24	28.42	59.64	99.85	100.40	101.50	99.40
42	90	42	90	42.46	90.42	42.08	90.16	101.09	100.46	100.19	100.17
56	120	56	120	56.09	120.08	56.47	120.45	100.16	100.06	100.83	100.37
70	150	70	150	69.64	149.85	70.66	150.37	99.48	99.90	100.94	100.24
84	180	84	180	84.15	180.18	83.54	180.47	100.17	100.10	99.45	100.26

SD Proposed method				SD Reference method			
Levofloxacin		Azithromycin		Levofloxacin		Azithromycin	
DDQ	*p*-CA	DDQ	*p*-CA	DDQ	*p*-CA	DDQ	*p*-CA
0.6692	0.3640	0.7416	0.3512	0.5690	0.3942	0.7302	0.3400

t-Test				F-test			
Levofloxacin		Azithromycin		Levofloxacin		Azithromycin	
DDQ	*p*-CA	DDQ	*p*-CA	DDQ	*p*-CA	DDQ	*p*-CA
0.2459	0.1243	0.0239	0.0500	0.7229	1.1728	0.9694	0.9372

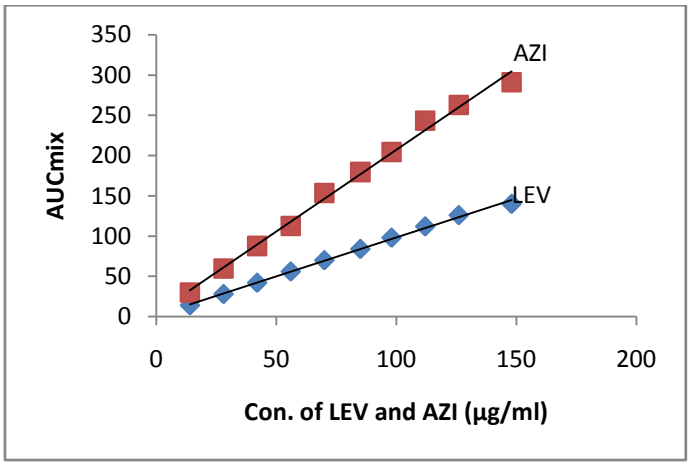

Fig 15: Plot of AUCmix vs Con. of LEV & AZI-DDQ in dosage form

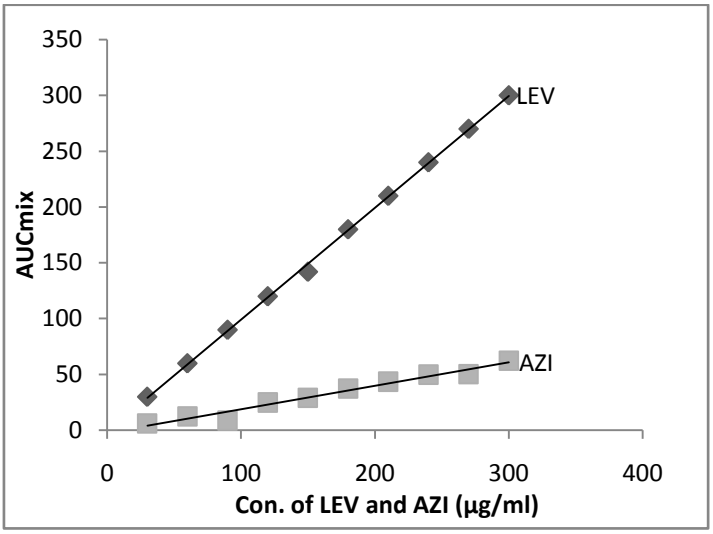

Fig 16: Plot of AUCmix vs Con. of LEV & AZI-p-CA in dosage form

V. CONCLUSION

A new way of analysis of mixed dosage forms using DDQ (Method A) and *p*-CA (Method B) involving the concept of area under curve is proposed, These methods are tested and validated. This is applied to the mixture of levofloxacin and azithromycin.

ACKNOWLEDGEMENTS

The authors are thankful to Prof. G. Venkateshwarlu, Department of Chemistry, Osmania University, Hyderabad for helpful discussion and to Sri M. Ravindra Reddy, Chairman, Managing Committee, SAP College, Vikarabad for providing facilities. The authors are thankful to the UGC for financial assistance under Major Research Project.

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T. Veeraiah. "Simultaneous spectrophotometric determination of Levofloxacin and Azithromycin using π-acceptors as analytical reagents." IOSR Journal of Pharmacy (IOSRPHR), vol. 9, no. 1, 2019, pp. 50-61.